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Targeting Human Papillomavirus Type 16 E7 to the Endosomal/Lysosomal Compartment Enhances the Antitumor Immunity of DNA Vaccines against Murine Human Papillomavirus Type 16 E7-Expressing Tumors

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ABSTRACT

DNA vaccination is an attractive approach for tumor immunotherapy because of its stability and simplicity of delivery. Advances demonstrate that helper T cell responses play a critical role in initiating immune responses. The aim of the current study is to test whether targeting HPV-16 E7 to the endosomal/lysosomal compartment can enhance the potency of DNA vaccines. We linked the lysosome-associated membrane protein 1 (LAMP-1) to HPV-E7 to construct a chimeric DNA, Sig/E7/LAMP-1 DNA. For *in vivo* tumor prevention experiments, mice were vaccinated with E7 DNA or Sig/E7/LAMP-1 DNA via gene gun, followed by tumor challenge. For *in vivo* tumor regression experiments, mice were first challenged with tumor cells and then vaccinated with E7-DNA or Sig/E7/LAMP-1 DNA. Intracellular cytokine staining with flow cytometry analysis, cytotoxic T lymphocyte (CTL) assays, enzyme-linked immunoabsorbent assay (ELISA), and enzyme-linked immunospot (ELISPOT) assays were used for *in vitro* E7-specific immunological studies. In both tumor prevention and tumor regression assays, Sig/E7/LAMP-1 DNA generated greater antitumor immunity than did wild-type E7 DNA. In addition, mice vaccinated with Sig/E7/LAMP-1 DNA had greater numbers of E7-specific $CD4^+$ helper T cells, higher E7-specific CTL activity, and greater numbers of $CD8^+$ T cell precursors than did mice vaccinated with Sig/E7 or wild-type E7 DNA. Sig/E7 generated a stronger E7-specific antibody response than did Sig/E7/LAMP-1 or wild-type E7 DNA. Our results indicate that linkage of the antigen gene to an endosomal/lysosomal targeting signal may greatly enhance the potency of DNA vaccines.

OVERVIEW SUMMARY

Naked DNA vaccines represent an attractive approach for tumor immunotherapy. In this study, we evaluated the effect of an endosomal–lysosomal targeting signal on the potency of naked DNA vaccines delivered intradermally via gene gun. We found that addition of the LAMP-1 endosomal/lysosomal sorting signal to the human papillomavirus type 16 E7 gene significantly increased the generation of tumor cell-specific $CD4^+$ helper T cells and $CD8^+$ cytotoxic T lymphocytes and, most importantly, increased the *in vivo* antitumor effect. The results indicate that linkage of the antigen gene to an endosomal/lysosomal targeting signal may greatly enhance the potency of DNA vaccines.

INTRODUCTION

NAKED DNA VACCINES have been reported as an attractive approach for generating antigen-specific vaccines because of their stability and simplicity of delivery. DNA vaccines possess several advantages over existing viral vector systems de-

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signed for *in vivo* gene delivery, even though such viral vectors are efficient in delivering genes of interest to targeted cells. For example, immune recognition after adenoviral vector or vaccinia delivery inhibits repeat vaccination with the same delivery system, while retroviral vectors display low *in vitro* infectivity and have potential virus-associated complications, including helper virus replication and insertional mutagenesis. In contrast, naked plasmid DNA is relatively safe and can be repeatedly administered. Furthermore, multiple DNA constructs can be administered together. Another advantage is that DNA vaccines can be easily prepared in large scale with high purity. Finally, DNA is highly stable relative to proteins and other biological polymers. As a vaccine, DNA has the unique ability either to integrate stably into the genome or to be maintained long term in an episomal form. This provides an opportunity for expression of the immunizing antigen over an extended period of time and enhancement of immunologic memory (for review, see Pardoll and Beckerleg, 1995; Donnelly *et al.*, 1997).

One of the concerns about DNA vaccines is their potency, since they do not have the intrinsic ability to amplify *in vivo* as viral vaccines do. Naked DNA also has no clear cell type specificity. It is therefore necessary to find an efficient route for administering DNA vaccines to appropriate target cells. Presentation of the antigen, which is encoded by the DNA vaccine, is best mediated by professional antigen-presenting cells (APCs). APCs are capable of taking up, processing, and presenting antigen to T cells in the context of costimulatory signals required for T cell activation. One of the best methods for delivering DNA vaccine to APCs is to use a gene gun to deliver DNA-coated gold beads to the epidermis (Fynan *et al.*, 1993). The gene gun can efficiently deliver DNA into epidermal MHC class II^+ bone marrow-derived APCs, also known as Langerhans cells, which move in the lymphatic system from bombarded skin to the draining lymph nodes (Condon *et al.*, 1996). Thus, cutaneous genetic immunization via gene gun is capable of directly introducing genes of interest into professional antigen-presenting cells *in vivo*. The potency of DNA vaccines delivered via gene gun can be further enhanced by strategies that help target antigens to both the MHC class I and class II processing pathways of the APCs.

It is clear that $CD4^+$ T cells are critical to the generation of most immune responses. In particular, increasing evidence points to the critical role of $CD4^+$ T cell responses in generating effective antitumor immunity (Golumbek *et al.*, 1991; Dranoff *et al.*, 1993; Ostrand-Rosenberg, 1994; Topalian *et al.*, 1994; Lin *et al.*, 1996). We previously described a molecular approach that directly routed a nuclear/cytoplasmic antigen, human papillomavirus (HPV) type 16 E7, into the endosomal and lysosomal compartments and enhanced the presentation of E7 antigen to MHC class II-restricted $CD4^+$ T cells (Wu *et al.*, 1995). This was accomplished by constructing a chimeric gene, Sig/E7/LAMP-1, in which E7 was linked to the endoplasmic reticulum translocation signal peptide (Sig) on its amino terminus and to the transmembrane and lysosomal targeting domains of the lysosome-associated membrane protein 1 (LAMP-1) on its carboxy terminus (Wu *et al.*, 1995). LAMP-1 is a type 1 transmembrane protein localized predominantly in lysosomes and late endosomes (Chen *et al.*, 1985; Lewis *et al.*, 1985). The cytoplasmic domain of LAMP-1 protein contains the amino acid sequence Tyr-Gln-Thr-Ile, which mediates the targeting of LAMP-1 into the endosomal and lysosomal compartments (Williams and Fukuda, 1990; Guarnieri *et al.*, 1993). This specific targeting of HPV-16 E7 to the endosomal and lysosomal compartments allows antigenic peptides of E7 to complex with MHC class II molecules and enhances MHC class II presentation (Wu *et al.*, 1995). Specfically, we showed that the Sig/E7/LAMP-1 recombinant vaccinia *in vivo* generated greater E7-specific antibody production and $CD4^+$ T cell-mediated lymphoproliferative responses than did vaccinia expressing the wild-type HPV-16 E7 gene (Wu *et al.*, 1995). In addition, E7-specific cytotoxic T lymphocyte (CTL) responses were augmented as well, possibly as a consequence of enhanced $CD4^+$ helper T cell function (Wu *et al.*, 1995).

We chose HPV-16 E7 as a model antigen for vaccine development because HPVs, particularly HPV-16, are associated with most cervical cancers. HPV oncogenic proteins E6 and E7 are coexpressed in most HPV-containing cervical cancers and are important in the induction and maintenance of cellular transformation. Therefore, vaccines or immunotherapies targeting E7 and/or E6 proteins may provide an opportunity to prevent and treat HPV-associated cervical malignancies. HPV-16 E7 is a well-characterized cytoplasmic/nuclear protein that is more conserved than E6 in HPV-associated cancer cells (for review, see Wu, 1994).

Targeting of antigens to the MHC class II processing compartment has two advantages. First, presentation of MHC class I and class II epitopes by the same APC provides a better opportunity for $CD4^+$ T cells to provide help during the priming of cytotoxic T lymphocyte (CTL) precursors. Second, antigen-specific $CD4^+$ helper T cells themselves have been shown to be important effectors for antitumor and other immune responses. If MHC class II-positive APCs are indeed the critical target for nucleic acid vaccines, linkage of antigen to an endosomal/lysosomal sorting signal could significantly enhance their potency. This hypothesis was tested by comparing DNA vaccines containing wild-type HPV-16 E7 with the Sig/E7/LAMP-1 chimera for immune response generation and their ability to protect animals against the E7-expressing murine model tumor (TC-1) (Lin *et al.*, 1996). In this study, we used cutaneous genetic immunization via gene gun to test our hypothesis.

MATERIALS AND METHODS

Murine tumor systems

Female C57BL/6 mice, 6 to 8 weeks in age, were purchased from the National Cancer Institute (Frederick, MD) and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). The animals were sacrificed by cervical dislocation at the time of tumor growth or at the end of an individual experiment. A murine cell line of C57BL/6 background, designated TC-1, was used. TC-1 cells were generated by cotransforming primary lung cells of C57BL/6 mice with HPV-16 E6 and E7 and activated *ras* oncogene (Lin *et al.*, 1996). The cells were cultured in RPMI 1640, supplemented with 10% (v/v) fetal bovine serum, penicillin–streptomycin (50 units/ml), 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 2 m*M* nonessential amino acids, and G418 (0.4 mg/ml) at 37°C with 5% CO_2.

On the day of tumor challenge, TC-1 cells were harvested by trypsinization, washed twice in Hanks' balanced salt solution, and resuspended in Hanks' balanced salt solution at the desired concentration for injection.

Plasmid DNA constructs and preparation

The Sig/E7/LAMP-1 chimeric gene was generated as described earlier (Wu, 1994). The chimeric gene and HPV-16 E7 gene were then cloned sequentially into the unique *Bam*HI and *Eco*RI cloning sites of the pCMVneoBam expression vector downstream of the cytomegalovirus promoter (Wu, 1994). Plasmid DNA including wild-type E7, Sig/E7, Sig/E7/LAMP-1, and the empty plasmid vector were transfected into subcloning efficient DH5α cells (Life Technologies, Gaithersburg, MD). DNA was then amplified and purified by using double CsCl purification (BioServe Biotechnologies, Laurel, MD). The integrity of plasmid DNA and the absence of *Escherichia coli* DNA or RNA were verified for each preparation by 1% agarose gel electrophoresis. DNA concentration was determined by the optical density at 260 nm. The presence of inserted E7, Sig/E7, or Sig/E7/LAMP-1 DNA fragments was confirmed by restriction enzyme digestion and gel electrophoresis.

DNA vaccination

DNA vaccination was delivered by gene gun ballistic bombardment, using methods similar to those described by Condon *et al.* (1996). In brief, DNA-coated gold particles were prepared by combining 25 mg of 1.6-μm gold beads (Bio-Rad, Hercules, CA) and 100 μl of 0.05 *M* spermidine (Sigma, St. Louis, MO). Plasmid DNA (50 μg) and 1.0 *M* $CaCl_2$ (100 μl) were added sequentially to the beads while mixing by vortex. This mixture was allowed to precipitate at room temperature for 10 min. The bead preparation was then centrifuged and washed three times in fresh absolute ethanol before resuspending in 3 ml of polyvinylpyrrolidone (0.1 mg/ml; Bio-Rad) in absolute ethanol. The solution was then loaded into tubing and allowed to settle for 4 min. The ethanol was gently removed and the beads were evenly attached to the sides of the tubing by rotation at 20 rpm for 4 min and drying. The latter was accomplished by flowing nitrogen gas into the tubing at a pressure of 4 lb/in^2. The dried tubing lined with beads was then cut into 0.5-in. sections (bullets) and stored in a capped dry bottle at 4°C. Each bullet contained 1 μg of plasmid DNA and 0.5 mg of gold. The DNA-coated gold particles (1 μg of DNA per bullet) were delivered to the shaved abdominal region of the mice, using a helium-driven gene gun (Bio-Rad) at a discharge pressure of 400 lb/in^2. In addition, mice vaccinated with uncoated gold particles were used as controls. Two weeks later, the mice were revaccinated by the same regimen used for the first vaccination.

Intracytoplasmic cytokine staining and flow cytometry analysis

To determine E7-specific $CD8^+$ CTL precursors and E7-specific $CD4^+$ helper T cell precursors, splenocytes from naive or vaccinated groups of mice were incubated either with the E7 peptide (amino acids [aa] 49–57) that contains an MHC class I epitope (Feltkamp *et al.*, 1993) or with the E7 peptide (aa 30–67) that contains an MHC class II epitope (Tindle *et al.*, 1991) at a concentration of 2 μg/ml for 20 hr. Splenocytes cultured without E7 peptides were used as controls. For intracytoplasmic cytokine staining, splenocytes were further treated with Golgistop (PharMingen, San Diego, CA) 6 hr before harvesting the cells from the culture, using the vendor protocol. Splenocytes were then washed once in washing buffer (1 × phosphate-buffered saline [PBS] containing 1% fetal bovine serum) and stained with phycoerythrin (PE)-conjugated monoclonal rat anti-mouse CD8 or CD4 antibody (PharMingen) at a concentration of 20 μg/ml for 20 min. The rat IgG_1 antibody (PharMingen) was used as immunoglobulin isotype control. Splenocytes were then subjected to intracellular cytokine staining, using the Cytofix/Cytoperm kit (PharMingen) and fluorescein isothiocyanate (FITC)-conjugated anti-interferon γ (IFN-γ) (20 μg/ml) or FITC-conjugated anti-interleukin 4 (IL-4) antibodies (20 μg/ml) (PharMingen) according to the manufacturer instructions. The IL-4-secreting activated mouse splenocytes (MiCK-2) from PharMingen were used as positive controls to assure the success of intracytoplasmic IL-4 staining for this study. Samples were acquired on a FACScan flow cytometer and data were analyzed using CellQuest software (Becton Dickson Immunocytometry Systems, San Jose, CA).

Enzyme-linked immunoabsorbent assay

Enzyme-linked immunosorbent assays (ELISAs) were performed according to the protocol described previously, with some modification (Wu *et al.*, 1995). On days 7 and 14 after the second vaccination, pooled tail vein blood samples were collected from each group of mice. The serum was used to detect antibodies against HPV-16 E7 by ELISA, using 96-microwell plates coated with yeast-derived HPV-16 E7 proteins as previously described (Wu *et al.*, 1995). Blood samples from mice that received intraperitoneal vaccinations of Sig/E7/LAMP-1 vaccinia (1 × 10^7 PFU/mouse) were used as positive controls.

Cytotoxic T lymphocyte assay

For the characterization of splenocytes from vaccinated mice, *in vitro* stimulation and measurement of cytotoxic T lymphocyte (CTL) activity were performed by the protocols described previously (Wu *et al.*, 1995). In brief, mouse splenocytes from each group were harvested on day 14 after vaccination. Splenocytes were cocultured with mitomycin-treated HPV-16 E7-expressing syngeneic TC-1 tumor cells (stimulators) for 6 days. CTL assays were performed in a standard 4-hr chromium release assay. Specific lysis was calculated as (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100%. In addition, to determine E7 peptide-specific CTL responses, the splenocytes of DNA-vaccinated mice were cultured for 6 days with 1 μg of E7-specific H-$2D^b$ CTL epitope (E7 49–57, RAHYNIVTF) per milliliter (Feltkamp *et al.*, 1993) and murine interleukin 2 (20 units/ml). A standard chromium release assay was performed against MC57G (H-2^b) cells pulsed with E7 (aa 49–57) and labeled with 0.2 mCi of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL). For the determination of MHC class I presentation of E7 by a dendritic cell line (Shen *et al.*, 1997) infected with various vaccinias, a standard chromium release assay was performed with dendritic cells (DCs) infected with 1 × 10^7 PFU of wild-type vaccinia, E7-

vaccinia, and Sig/E7/LAMP-1 vaccinia (Wu *et al.*, 1995) for 6 hr and labeled with 0.2 mCi of $Na_2{}^{51}CrO_4$ (Amersham). Untransfected DCs were used as a negative control. We used a serial dilution of E7-specific T cells (beginning with an enhancer-to-target [*E:T*] ratio of 3:1) incubated with 5×10^3 ^{51}Cr-labeled DCs that were either infected with various vaccinia or uninfected. Specific lysis was calculated as described earlier.

Generation of an E7-specific CTL cell line

Six-week-old female C57BL/6 (*H-2^b*) mice were immunized by intraperitoneal injection of 10^7 PFU of vaccinia-Sig/E7/LAMP-1. Splenocytes were harvested on day 8. For initial *in vitro* stimulation, 4×10^6 splenocytes were pulsed with IL-2 at a concentration of 20 U/ml and 1 μM E7 peptide (amino acids 49–57) for 6 days. Propagation of the E7-specific CTL cell line was performed in 24-well plates by mixing (2 ml/well) 1×10^6 splenocytes containing E7-specific CTLs with 3×10^6 irradiated splenocytes and pulsing them with IL-2 at a concentration of 20 U/ml and 1 μM E7 peptide (amino acids 49–57). This procedure was repeated every 6 days. The specificity of the E7 CTL line was characterized by the CTL assay. Flow cytometry was performed to demonstrate the expression of the CD8 marker.

Enzyme-linked immunospot assays for IFN-γ-secreting cells

The enzyme-linked immunospot (ELISPOT) assays described by Miyahira *et al.* were modified to detect HPV-16 E7-specific T cell precursors (Miyahira *et al.*, 1995). The 96-well filtration plates (Millipore, Bedford, MA) were coated with rat anti-mouse IFN-γ antibody (clone R4-6A2, 10 μg/ml; PharMingen) in 50 μl of PBS. After overnight incubation at 4°C, the wells were washed and blocked with culture medium containing 10% fetal bovine serum. For the detection of E7-specific CD4$^+$ and CD8$^+$ T cell precursors in vaccinated mice, splenocytes from each group were harvested on day 14 after vaccination. Serial dilutions of fresh isolated splenocytes from each vaccinated group of mice, starting from 1×10^6/well, were added to the wells along with interleukin 2 (15 units/ml). Cells were incubated at 37°C for 24 hr either with or without 1 μg of E7-specific H-2D^b CTL epitope E7 (aa 49–57) per milliliter (Feltkamp *et al.*, 1993). For the *in vitro* demonstration of MHC class I presentation of E7 in DCs, we infected DCs (Shen *et al.*, 1997) with various vaccinias (used as protein expression vectors). We used 1×10^7 PFU of wild-type vaccinia, E7-vaccinia, and Sig/E7/LAMP-1 vaccinia (Wu *et al.*, 1995) to infect 2×10^6 targeted DCs for 16 hr, and used untransfected DCs as a negative control. Serial dilutions of E7-specific T cells (beginning with 800 cells/well) were cultured with 1×10^5 DCs infected with the various vaccinias. After culture, the plate was washed and then incubated with biotinylated IFN-γ antibody (clone XMG1.2, 5 μg/ml; PharMingen) in 50 μl of 1× PBS overnight at 4°C. After six washes, avidin–alkaline phosphatase (1.25 μg/ml; Sigma) in 50 μl of 1× PBS was added and the plate was incubated for 2 hr at room temperature. After washing, spots were developed by adding 50 μl of 5-bromo-4-chloro-3-indolylphosphate toluidinium/nitroblue tetrazolium (BCIP/NBT) solution (Boehringer Mannheim, Indianapolis, IN) and incubating the plate at room temperature for 1 hr. The spots were counted with a dissecting microscope.

In vivo *tumor protection experiments*

Two sets of tumor protection experiments were done. For the first tumor protection experiment, mice (eight per group) were vaccinated via gene gun with 2 μg of Sig/E7/LAMP-1 DNA, E7 DNA, or the control plasmid without insert. Two weeks later, the mice were revaccinated according to the same regimen used for the first vaccination. In total, each mouse received 4 μg of plasmid DNA. Two weeks after the second vaccination, mice were challenged with TC-1 tumor cells at a dose of 1×10^5 cells per mouse by subcutaneous injection on the left flank. In addition, eight unvaccinated mice received the same amount of TC-1 cells for the natural tumor growth control. Tumor growth was monitored by visual inspection and palpation twice weekly. On day 40 postchallenge, the tumor-free mice were rechallenged by injecting 1×10^5 TC-1 cells per mouse on the right flank, followed by a tumor check twice a week. Five naive mice received the same amount of TC-1 cells and served as a control.

For the second tumor protection experiment, mice (five per group) were vaccinated with 1 μg of either Sig/E7/LAMP-1 DNA or wild-type E7 DNA via gene gun. An additional group of five mice received pure uncoated gold beads as a control. Two weeks later, the vaccinated mice were challenged with 2×10^4 TC-1 cells per mouse subcutaneously on the left flank. Tumor growth was monitored by visual inspection and palpation twice weekly.

In vivo *tumor treatment experiments*

Tumor cells for injection and DNA vaccines for immunization were prepared as described above. First, 2×10^4 TC-1 cells were injected subcutaneously in the left leg. Three and 10 days after challenge with TC-1 tumor cells, mice were given 2 μg of Sig/E7/LAMP-1 DNA, E7 DNA, or control plasmid via gene gun. Five mice were used for each DNA vaccine. Each mouse received a total of 4 μg of Sig/E7/LAMP-1 DNA, E7 DNA, or control plasmid DNA. Mice were monitored twice weekly and euthanized after the development of tumors.

RESULTS

DNA vaccines encoding HPV-16 E7 with LAMP-1 sorting signal enhance E7-specific CD4$^+$ helper T type 1 immune responses

We have previously shown that the Sig/E7/LAMP-1 recombinant vaccinia *in vivo* generated greater CD4$^+$ T cells-mediated lymphoproliferative responses than did vaccinia expressing the wild-type HPV-16 E7 gene (Wu *et al.*, 1995). In this study, we further investigated whether the Sig/E7/LAMP-1 DNA vaccine administered via gene gun resulted in enhanced E7-specific MHC class II-restricted CD4$^+$ T cell responses. Differentiation of precursor helper T cells into helper T type 1 (Th1) or Th2 cells might have important biological implications in the generation of antitumor immunity. Therefore, we performed double staining for the CD4 surface marker and intra-

cellular IFN-γ or IL-4 on splenocytes from immunized mice followed by flow cytometry analysis to define distinct immunological roles of our recombinant DNA vaccine in inducing Th1 versus Th2 responses. For the detection of the E7-specific IFN-γ-secreting $CD4^+$ T cell, splenocytes from immunized mice were cultured *in vitro* with the E7 peptide (aa 30–67) overnight and stained for both CD4 and intracellular IFN-γ. The E7 peptide (aa 30–67) contains a major helper T cell epitope in the E7 open reading frame of the human papilloma virus (HPV) type 16 (Tindle *et al.*, 1991). The percentage of IFN-γ-secreting $CD4^+$ T cells was analyzed by flow cytometry. As shown in Fig. 1A, mice vaccinated with Sig/E7/LAMP-1 DNA generated the most $CD4^+IFN\text{-}\gamma^+$ double-positive cells. The relative frequency of E7-specific IFN-γ-secreting $CD4^+$ T cells was plotted in Fig. 1B. Mice vaccinated with Sig/E7/LAMP-1 DNA vaccine generated the highest IFN-γ-secreting E7-specific $CD4^+$ helper T cell precursors [137.0 ($\pm$9.9)/10^6 splenocytes]. In addition, we observed a less dramatic increase in E7-specific IFN-γ-secreting E7-specific $CD4^+$ helper T cell precursors [79.5 ($\pm$6.4)/10^6 splenocytes] in mice vaccinated with Sig/E7 DNA. No significant enhancement of the E7-specific $CD4^+$ T cell was observed in mice vaccinated with wild-type E7 DNA [31.0 ($\pm$7.1)/10^6 splenocytes], control plasmid DNA [16.5 ($\pm$3.5)/10^6 splenocytes], or naive mice [16.5 ($\pm$3.5)/10^6 splenocytes].

We then analyzed IL-4-secreting E7-specific $CD4^+$ T cells in mice vaccinated with various DNA vaccines. IL-4-secreting activated mouse splenocytes (MiCK-2; PharMingen) were used as positive controls to assure the success of intracellular IL-4 staining for this study. The specificity of the IL-4 staining was demonstrated by the absence of $CD4^+IL\text{-}4^+$ cells when the IL-4 antibody was omitted (Fig. 2A, top). As shown in Fig. 2A (bottom), no significant $CD4^+IL\text{-}4^+$ double-positive cells were identified in the mice vaccinated with Sig/E7/LAMP-1 SNA, Sig/E7 DNA, wild-type E7 DNA, or plasmid DNA vaccination or in the naive mice without vaccination. In addition, no significant variation was observed in the frequency of IL-4-secreting $CD4^+IL\text{-}4^+$ T cells in the various vaccination groups (Fig. 2B).

While no significant $CD4^+IL\text{-}4^+$ double-positive cells were identified in mice vaccinated with Sig/E7/LAMP-1 DNA (Fig. 2), significant numbers of $CD4^+IFN\text{-}\gamma^+$ double-positive cells were observed in mice vaccinated with Sig/E7/LAMP-1 DNA (Fig. 1). These results indicated that mice vaccinated with Sig/E7/LAMP-1 DNA vaccine generated E7-specific Th1-type cytokine profiles. These results were further confirmed by ELISAs, using the supernatants of splenocytes from the vaccinated mice. The splenocytes were stimulated *in vitro* with E7 protein and assayed for the presence of IFN-γ and IL-4. The highest IFN-γ level was found in mice vaccinated with

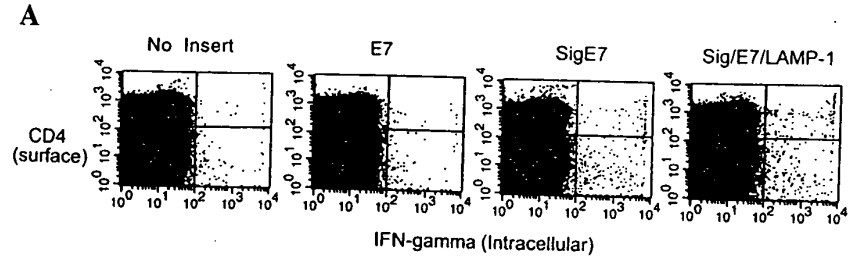


FIG. 1. Flow cytometry analysis of IFN-γ-secreting E7-specific $CD4^+$ cells in mice vaccinated with various recombinant DNA vaccines. **(A)** Splenocytes from vaccinated mice were cultured *in vitro* with E7 peptide (aa 30–67) overnight and stained for both CD4 and intracellular IFN-γ. The percentage of IFN-γ-secreting $CD4^+$ T cells was analyzed by flow cytometry. Mice vaccinated with the Sig/E7/LAMP-1 DNA generated the greatest E7-specific $CD4^+$ helper T cell precursors. **(B)** The number of IFN-γ-producing E7-specific $CD4^+$ T cells was determined by flow cytometry in the presence (solid columns) and absence (open columns) of E7 peptide (aa 30–67). One set of experiments is shown and similar results were observed in three other sets of experiments. Data are expressed as the mean number of $CD4^+IFN\text{-}\gamma^+$ cells per 10^6 splenocytes $\pm$ SEM.

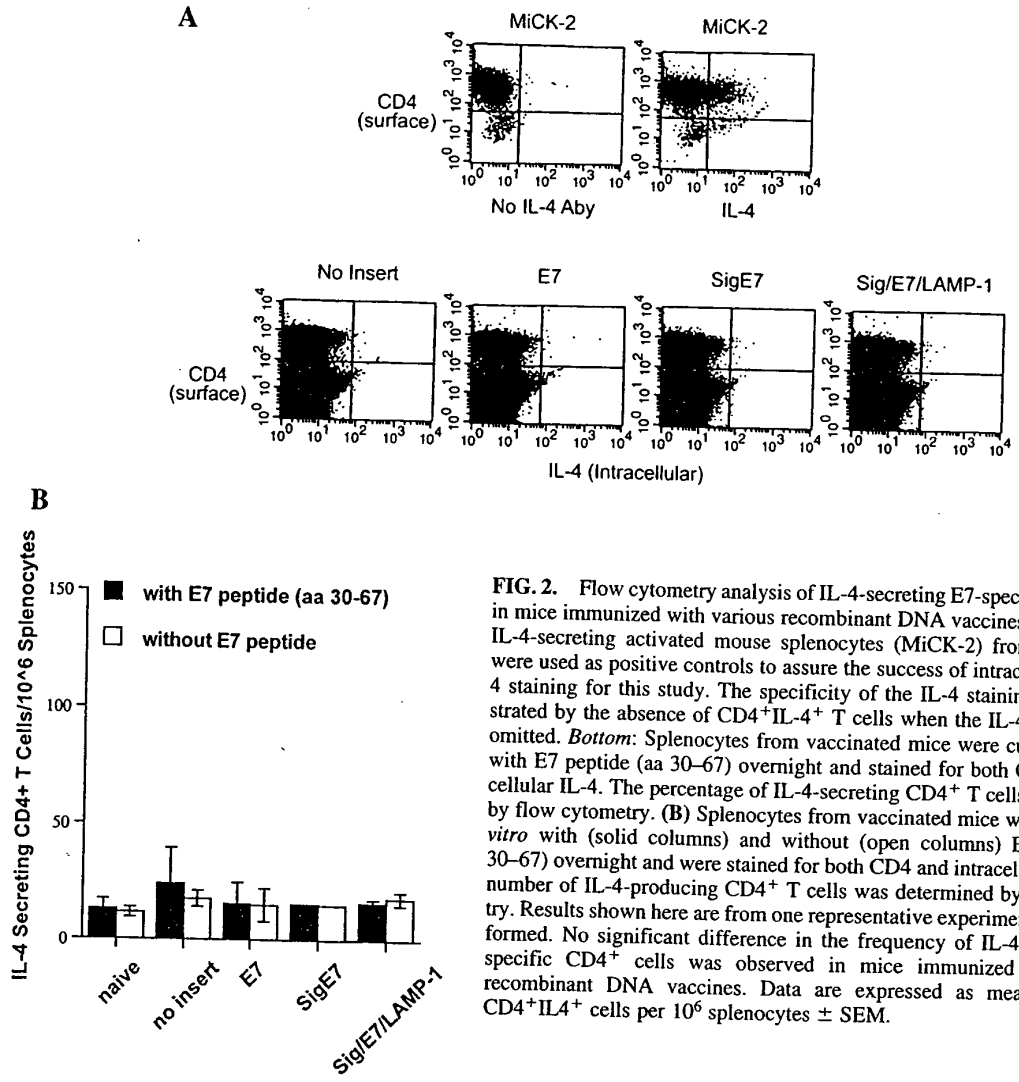


FIG. 2. Flow cytometry analysis of IL-4-secreting E7-specific $CD4^+$ cells in mice immunized with various recombinant DNA vaccines. **(A)** *Top*: The IL-4-secreting activated mouse splenocytes (MiCK-2) from PharMingen were used as positive controls to assure the success of intracytoplasmic IL-4 staining for this study. The specificity of the IL-4 staining was demonstrated by the absence of $CD4^+IL\text{-}4^+$ T cells when the IL-4 antibody was omitted. *Bottom*: Splenocytes from vaccinated mice were cultured *in vitro* with E7 peptide (aa 30–67) overnight and stained for both CD4 and intracellular IL-4. The percentage of IL-4-secreting $CD4^+$ T cells was analyzed by flow cytometry. **(B)** Splenocytes from vaccinated mice were cultured *in vitro* with (solid columns) and without (open columns) E7 peptide (aa 30–67) overnight and were stained for both CD4 and intracellular IL-4. The number of IL-4-producing $CD4^+$ T cells was determined by flow cytometry. Results shown here are from one representative experiment of three performed. No significant difference in the frequency of IL-4-secreting E7-specific $CD4^+$ cells was observed in mice immunized with various recombinant DNA vaccines. Data are expressed as mean number of $CD4^+IL4^+$ cells per 10^6 splenocytes ± SEM.

Sig/E7/LAMP-1 DNA. IL-4 could not be detected in the supernatant of any vaccinated group (data not shown).

Vaccination with Sig/E7/LAMP-1 DNA generates high E7-specific antibody responses

The HPV-16 E7-specific antibody responses in the sera of the vaccinated mice were determined by a direct ELISA on days 7 and 14 after the second vaccination. As shown in Fig. 3, 7 days after the second vaccination, we detected a significant E7-specific antibody response only in the sera of mice vaccinated with Sig/E7 DNA. However, 14 days after the second vaccination, significant E7-specific antibody responses were observed in the sera of both Sig/E7/LAMP-1- and Sig/E7 DNA-vaccinated mice, with the Sig/E7 DNA-vaccinated mice generating higher E7-specific antibody responses. In contrast, no E7-specific antibody responses could be detected in the sera of the mice vaccinated with either wild-type E7 DNA or the "empty" plasmid (Fig. 3). These results indicated that mice vaccinated with Sig/E7/LAMP-1 and Sig/E7 DNA are capable of generating E7-specific antibody responses, with Sig/E7 generating earlier and greater responses.

DNA vaccines encoding HPV-16 E7 with LAMP-1 sorting signal enhance E7-specific $CD8^+$ cytotoxic T cell immune responses

Since $CD8^+$ cytotoxic T cells have been implicated as important effector cells for antitumor effects, we used the CTL assay to study the E7-specific CTL activity. For the CTL assay, the E7 DNA generated higher CTL activity than did the control plasmid. However, the highest CTL activities were indeed observed in mice vaccinated with Sig/E7/LAMP-1 DNA vaccine when E7-expressing TC-1 tumor cells were used as target cells for CTL assays (Fig. 4). Similarly, when E7 peptide (aa 49–57)-pulsed MC57G cells were used as target cells for CTL assays, mice vaccinated with Sig/E7/LAMP-1 DNA vaccine also generated the highest CTL activities (data not shown).

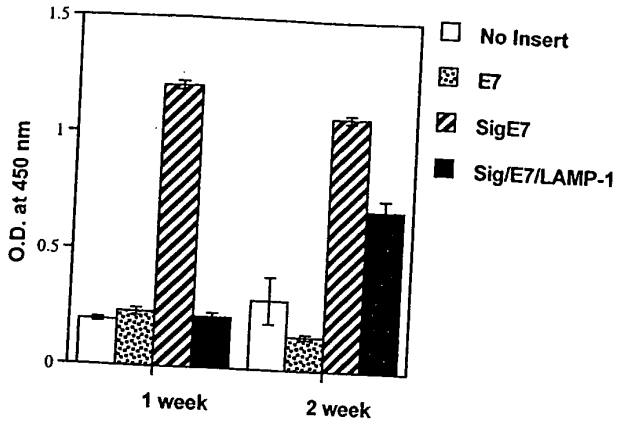


FIG. 3. E7-specific antibody responses in C57BL/6 mice immunized with various recombinant DNA vaccines. C57BL/6 mice were immunized with control plasmid (no insert), wild-type E7, Sig/E7, or Sig/E7/LAMP-1 DNA via gene gun. Serum samples were obtained from immunized mice 7 and 14 days after the second vaccination. The presence of the E7-specific antibody was detected by ELISA, using serial dilutions of sera. The results from the 1:100 dilution are presented, showing mean absorbance (OD) at 450 nm ± SEM.

Next, we used the ELISPOT assay to determine the precursor frequencies of E7-specific $CD8^+$ T cells in each vaccinated group. The IFN-γ secretion by individual $CD8^+$ T cells is visualized as spots, which were revealed with enzyme-labeled anti-IFN-γ monoclonal antibodies. ELISPOT assays provide a quantitative assessment of the number of activated antigen-specific $CD8^+$ T cells (Murali-Krishna *et al.*, 1998). As shown in Fig. 5, the highest ELISPOT numbers were observed in mice vaccinated with Sig/E7/LAMP-1 DNA vaccine [82.6 ($\pm$3.9/3 $\times$ 10^5 splenocytes]. We also observed that Sig/E7 DNA generated a slight increase in the number of $CD8^+$ T cell precursors [48.0 ($\pm$2.0)/3 $\times$ 10^5 splenocytes]. The E7-specific $CD8^+$ T cell precursors can be further determined by flow cytometry analysis, using double staining for CD8 and intracellular IFN-γ. As shown in Fig. 6, mice vaccinated with Sig/E7/LAMP-1 DNA generated the highest E7-specific $CD8^+$

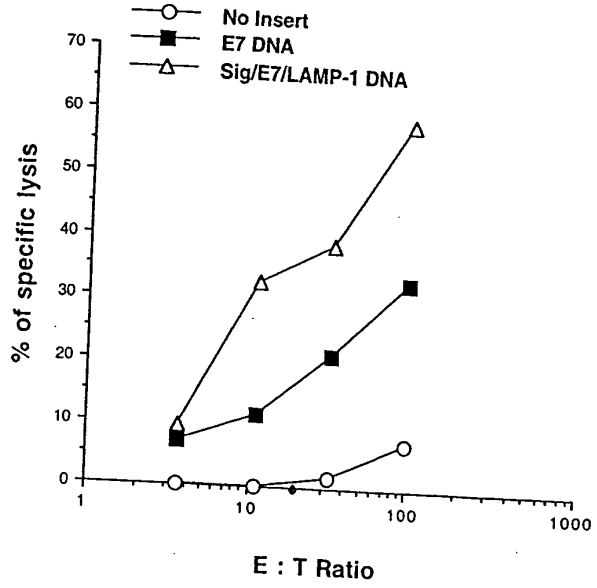


FIG. 4. CTL responses in C57BL/6 mice immunized with various recombinant DNA vaccines. C57BL/6 mice were immunized with control plasmid (open circles), wild-type E7 DNA (closed squares), or Sig/E7/LAMP-1 DNA (open triangles) intradermally via gene gun. Splenocytes were harvested 2 weeks after DNA vaccination. Splenocytes were stimulated *in vitro* with mitomycin-treated TC-1 tumor cells for 6 days before ^{51}Cr release assays were performed. TC-1 cells were used as target cells. Target cell-specific lysis was determined as described in Materials and Methods.

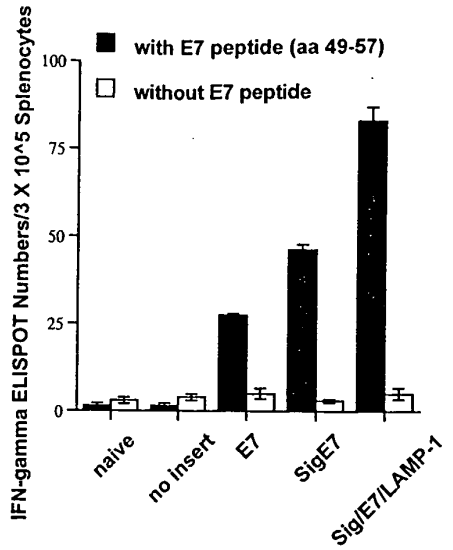


FIG. 5. Demonstration of E7-specific $CD8^+$ T cell precursors in C57BL/6 mice immunized with various recombinant DNA vaccines, using ELISPOT assays. C57BL/6 mice were vaccinated with control plasmid, wild-type E7, Sig/E7, or Sig/E7/LAMP-1 DNA intradermally via gene gun, or were not vaccinated. For vaccinated mice, 2 μg of DNA per mouse was given twice. Splenocytes were harvested 2 weeks after vaccination. The number of IFN-γ-producing E7-specific T cell precursors was determined in the presence (solid columns) and absence (open columns) of the E7 peptide (aa 49–57). Data are expressed as mean number of spots per 3×10^5 splenocytes $\pm$ SEM. Results shown here are from one representative experiment of three performed.

T cell precursors [110.0 ($\pm$7.1)/3 $\times$ 10^5 splenocytes]. Mice vaccinated with Sig/E7 DNA generated a significant increase in E7-specific $CD8^+$ T cell precursors as well [92.5 ($\pm$3.5)/3 $\times$ 10^5 splenocytes]. The number of E7-specific $CD8^+$ T cell precursors generated by E7 DNA vaccination was 43.0 ($\pm$1.4)/3 $\times$ 10^5 splenocytes. In general, the number of E7-specific $CD8^+$ T cell precursors determined by flow cytometry using double staining for CD8 and intracytoplasmic IFN-γ was higher than that determined by the ELISPOT assays. With these three different methods (CTL assays, ELISPOT assays, and intracellular cytokine stainings), we have demonstrated that Sig/E7/LAMP-1 DNA immunization generated the highest E7-specific CTL responses. Mice vaccinated with Sig/E7/LAMP-1 DNA generated at least twofold higher E7-specific $CD8^+$ T cell precursors than did mice vaccinated with wild-type E7 DNA.

Dendritic cells expressing Sig/E7/LAMP-1 present E7 antigen through the MHC class I pathway

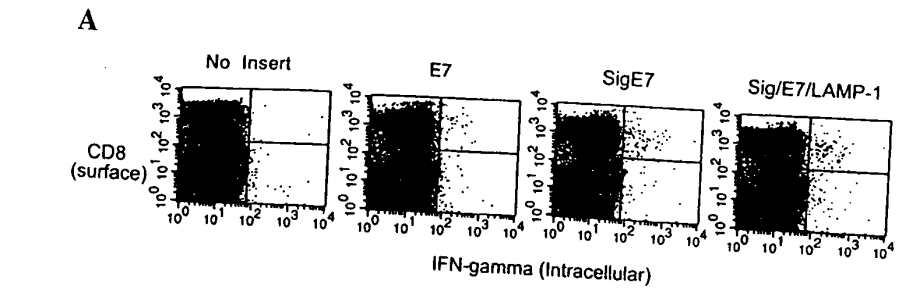
Since we observed greater numbers of enhanced $CD8^+$ T cell precursors in mice vaccinated with Sig/E7/LAMP-1, it is important to demonstrate if antigen-presenting cells expressing Sig/E7/LAMP-1 presented E7 through the MHC class I pathway. We used the ELISPOT and CTL assays to demonstrate MHC class I presentation of E7 antigen in dendritic cells infected with either Sig/E7/LAMP-1, wild-type E7, or wild-type vaccinia. An E7-specific T cell line was used as a readout for this assay. For the ELISPOT assay (Fig. 7), DCs expressing Sig/E7/LAMP-1 and wild-type E7 generated a noticeable number of spots with increasing numbers of E7-specific CTLs, while DCs expressing wild-type vaccinia generated a negligible number of spots, similar to control DCs. In addition, DCs expressing Sig/E7/LAMP-1 generated a higher number of spots compared with DCs expressing wild-type E7. Furthermore, the CTL assay confirmed the results generated from the ELISPOT assay. If MHC class I presentation of E7 occurred in DCs expressing Sig/E7/LAMP-1, one would expect specific lysis of these DCs by E7-specific $CD8^+$ T cells. As shown in Fig. 8, DCs infected with Sig/E7/LAMP-1 generated the highest percent specific lysis compared with DCs infected with other vaccinia. These results indicated that DCs expressing Sig/E7/LAMP-1 and wild-type E7 are capable of presenting E7 antigen through the MHC class I pathway. However, DCs expressing Sig/E7/LAMP-1 are more efficient at presenting E7 through the MHC class I pathway compared with DCs expressing wild-type E7.

DNA vaccines encoding HPV-16 E7 with LAMP-1 sorting signal protect mice against E7-expressing tumors

To determine whether the enhanced immune responses generated by incorporation of the LAMP-1 sorting signal into the DNA construct resulted in enhanced antitumor activity, we compared the ability of Sig/E7/LAMP-1 DNA and E7 DNA vaccines to protect animals against challenge with the E7-expressing TC-1 tumor. Two *in vivo* tumor protection experiments were performed with different doses of DNA vaccine. For the first experiment, mice were vaccinated with 2 μg of DNA per mouse and revaccinated 2 weeks later. Both Sig/E7/LAMP-1 DNA and E7 DNA vaccines generated potent antitumor immunity against E7 expressing TC-1 tumor. As shown in Fig. 9A, both Sig/E7/LAMP-1 DNA and E7 DNA vaccines generated 100% tumor protection for up to 8 weeks, in contrast to no tumor protection in unvaccinated mice (Fig. 9A). Interestingly, the no-insert plasmid backbone also generated 25% tumor protection. The antitumor immunity appeared to be long-lasting. When the tumor-free animals were rechallenged with 1×10^5 TC-1 cells per mouse subcutaneously on the opposite flank, no tumor growth was observed for up to 4 weeks after tumor rechallenge (data not shown). To compare the potency difference between E7 DNA and Sig/E7/LAMP-1 DNA, we decreased the vaccination dose in the second *in vivo* tumor protection experiment. Mice were vaccinated once with a dose of 1 μg of DNA per mouse. As shown in Fig. 9B, 60% of the mice vaccinated with Sig/E7/LAMP-1 DNA remained tumor free 60 days after tumor injection. In comparison, none of the mice vaccinated with wild-type E7 DNA remained tumor free. Furthermore, all of the mice inoculated with pure gold particles showed progressive tumor growth 2 weeks after tumor challenge (Fig. 9B).

DNA vaccines encoding HPV-16 E7 with LAMP-1 sorting signal eradicate previously established E7-expressing tumors in mice

To test the efficacy of DNA vaccines in eradicating established TC-1 tumors, TC-1 cells were first injected into



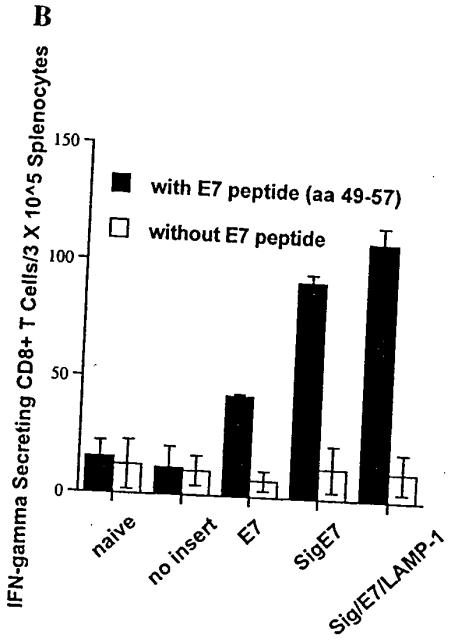


FIG. 6. Flow cytometry analysis of IFN-γ-secreting E7-specific $CD8^+$ cells in mice immunized with various recombinant DNA vaccines. **(A)** Splenocytes from vaccinated mice were cultured *in vitro* with the E7 peptide (aa 49–57) overnight and were stained for both CD8 and intracellular IFN-γ. The number of IFN-γ-secreting $CD8^+$ T cell precursors in mice immunized with various recombinant DNA vaccines was analyzed by flow cytometry. Mice vaccinated with the Sig/E7/LAMP-1 DNA generated the greatest number of E7-specific $CD8^+$ T cell precursors. **(B)** The number of IFN-γ-producing E7-specific $CD8^+$ T cells was determined by flow cytometry in the presence (solid columns) and absence (open columns) of E7 peptide (aa 49–57). Results shown here are from one representative experiment of three performed. Data are expressed as the mean number of $CD8^+IFN\text{-}\gamma^+$ cells per 3×10^5 splenocytes ± SEM.

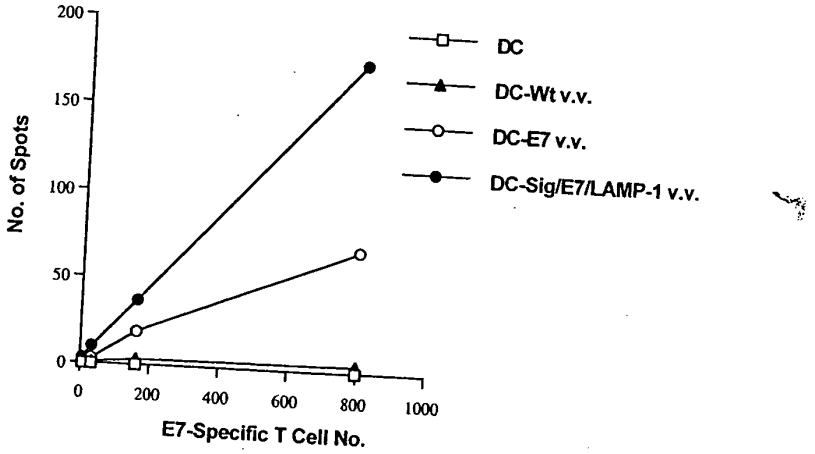


FIG. 7. Demonstration of MHC class I presentation of the E7 peptide in dendritic cells infected with various recombinant vaccinia, using the ELISPOT assay. A total of 1×10^7 PFU of wild-type vaccinia (filled triangles), wild-type E7 vaccinia (open circles), and Sig/E7/LAMP-1 vaccinia (filled circles) was used to infect targeted DCs (Shen *et al.*, 1997) for 16 hr, and untransfected DCs (open squares) were used as a negative control. Serial dilutions of E7-specific T cells (beginning with 800 cells/well) were cocultured with 1×10^5 DCs that were either infected with various vaccinia or uninfected. IFN-γ secretion by activated T cells was detected by this assay.

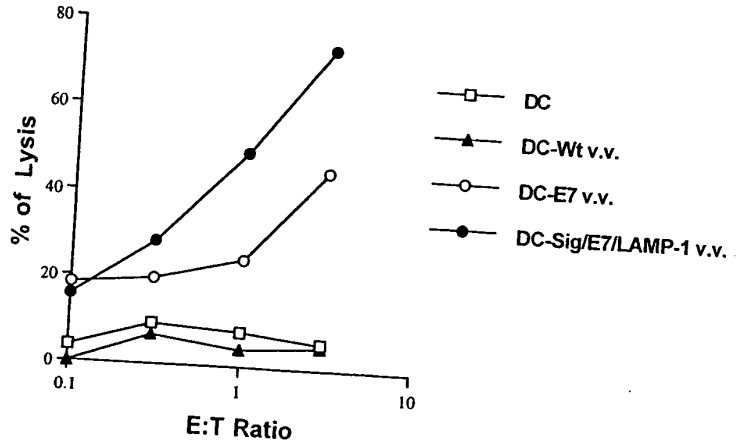


FIG. 8. Demonstration of MHC class I presentation of E7 in dendritic cells infected with various recombinant vaccinia, using the CTL assay. A total of 1×10^7 PFU of wild-type vaccinia (filled triangles), wild-type E7 vaccinia (open circles), and Sig/E7/LAMP-1 vaccinia (filled circles) was used to infect targeted DCs (Shen *et al.*, 1997) for 6 hr, and untransfected DCs (open squares) were used as a negative control. We used serial dilutions of E7-specific T cells (beginning with an *E:T* ratio of 3:1) incubated with 5×10^3 ^{51}Cr-labeled DCs that were either infected with various vaccinias or uninfected. The ^{51}Cr release assay was performed as described in Materials and Methods.

C57BL/6 mice subcutaneously at a dose of 2×10^4 cells per mouse in the left leg. Three days later, each mouse was treated with 2 μg of either control plasmid DNA, wild-type E7 DNA, or Sig/E7/LAMP-1 DNA intradermally with the gene gun. Ten days later, mice were revaccinated with the same dose and by the same method. As shown in Fig. 10, 100% of mice that received Sig/E7/LAMP-1 DNA vaccination remained tumor free 40 days after TC-1 challenge, whereas all of the unvaccinated mice and mice receiving the control plasmid developed tumor growth within 21 days after tumor challenge. In mice vaccinated with wild-type E7 DNA, a partial treatment effect was observed, with 60% of mice remaining tumor free 40 days after TC-1 challenge (Fig. 10).

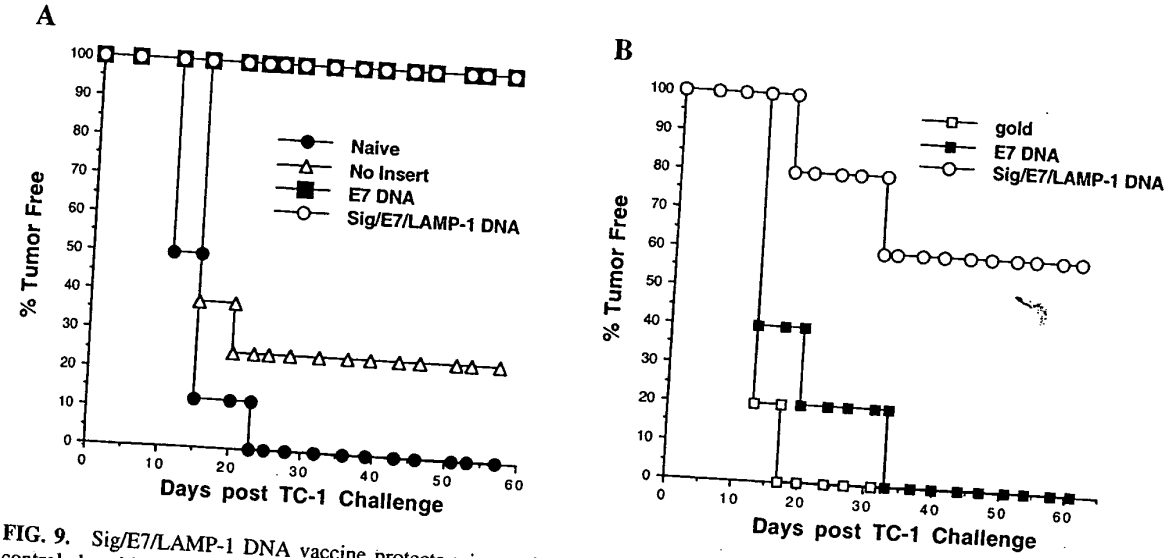


FIG. 9. Sig/E7/LAMP-1 DNA vaccine protects mice against challenge with TC-1 tumor. (**A**) Each mouse received 2 μg of control plasmid, wild-type E7, or Sig/E7/LAMP-1 DNA; 2 weeks later, the mice were revaccinated with the same type and amount of DNA as in the first vaccination. Two weeks after the second vaccination, mice were challenged subcutaneously with 1×10^5 TC-1 cells per mouse. The mice were monitored for evidence of tumor growth by palpation and inspection twice a week. (**B**) C57BL/6 mice either received uncoated gold particles or were immunized intradermally with E7 DNA or Sig/E7/LAMP-1 DNA at 1 μg per mouse. Two weeks later, mice were challenged subcutaneously with TC-1 tumor cells at 2×10^4 cells per mouse. Tumor growth was assessed by visual inspection and palpation twice per week.

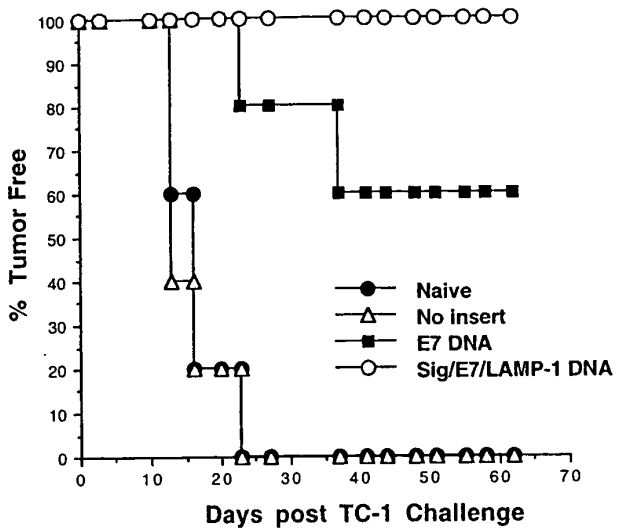


FIG. 10. Sig/E7/LAMP-1 DNA vaccine eradicates preexisting TC-1 tumor cells. Each mouse was initially challenged with 2×10^4 TC-1 cells subcutaneously, followed by DNA vaccination via gene gun 3 and 10 days after tumor challenge. The vaccination dose was 2 μg of DNA per mouse for each vaccination. The tumor growth was evaluated twice weekly by visual inspection and palpation.

DISCUSSION

In this study we have demonstrated that rerouting of a cytosolic tumor antigen into the endosomal/lysosomal compartment can profoundly improve the *in vivo* therapeutic potency of nucleic acid vaccines. A similar strategy may be applied to generate DNA vaccines for other tumor-specific antigens. The data we obtained about DNA vaccines support a previous study that we had conducted with vaccinia vectors, which found that use of the Sig/E7/LAMP-1 vaccinia vaccine generated stronger antitumor effects in C57BL/6 mice compared with wild-type E7 vaccinia vaccine (Lin *et al.*, 1996). However, even though vaccinia vectors are effective in generating a strong E7-specific antitumor effect, many individuals had previously been exposed to the vaccinia for smallpox immunization. We found that previous exposure to vaccinia can significantly limit the potency of the Sig/E7/LAMP-1 vaccinia vaccine (T.-C. Wu *et al.*, unpublished, 1999). The use of naked DNA vaccines circumvents the problem of immune responses to the vaccinia vaccine vector, which can result in rapid elimination of transduced cells or limit the effectiveness of readministration. Also, DNA vaccines do not have the potential health hazards of viral vectors.

The LAMP-1 targeting strategy has been successfully used in several other biological systems. For example, LAMP-1-mediated targeting of the HIV-1 envelope protein (gp160) to the endosomal/lysosomal compartment enhances its presentation to MHC class II-restricted T cells (Rowell *et al.*, 1995). Moreover, mice vaccinated with recombinant vaccinia expressing gp160/LAMP-1 generated greater gp160-specific lymphoproliferation responses and higher titers of anti-V3 loop antibodies than did mice vaccinated with recombinant vaccinia expressing wild-type gp160 (Ruff *et al.*, 1997). The LAMP-1 strategy has also been used to enhance MHC class II presentation in dendritic cells. Nair *et al.* demonstrated that dendritic cells transfected with the carcinoembryonic antigen (CEA) and LAMP-1 chimeric RNA construct can efficiently stimulate the CEA-specific $CD4^+$ T cells from human peripheral blood lymphocytes *in vitro* (Nair *et al.*, 1998). More recently, DNA vaccines based on the LAMP-1 targeting strategy have been applied to the HER-2/neu model antigen in a murine breast cancer model and generated a more potent vaccine effect than did unmodified HER-2/neu DNA vaccines (E. Jaffee, personal communication, 1999). These studies suggest the potential benefit of using the LAMP-1 targeting strategy in enhancing vaccine potency.

In our study, we have observed that vaccination with Sig/E7/LAMP-1 DNA via gene gun augmented E7-specific $CD4^+$ helper T cell type 1 immune responses (Figs. 1 and 2). The Th1-type cytokine profiles may result from the intradermal administration of the bacteria-derived DNA vaccines that we used in our study. Vaccination with bacteria-derived naked DNA has been reported to elicit cellular and humoral immune responses that have a helper T cell type 1 bias (Sato *et al.*, 1996). Injection of bacteria-derived naked DNA into murine dermis could lead to enhanced expression of MHC class II and costimulatory molecules by Langerhans cells in the overlying epidermis and intracytoplasmic IL-12 accumulation in a subpopulation of activated Langerhans cell (Jakob *et al.*, 1998). Therefore, the bacteria-derived naked DNA vaccines used in this study may preferentially elicit Th1-predominant immune responses. Several studies have indicated that the Th1-type immune response is one of the major components contributing to the development of T cell-mediated immunity against HPV-associated neoplasms (Tsukui *et al.*, 1996; Clerici *et al.*, 1997).

The increased understanding of the pathways for antigen presentation creates the potential for designing novel strategies to enhance vaccine potency. For example, a DNA vaccine encoding a fusion antigen that is directed to sites of immune induc-

tion has been shown to enhance vaccine potency (Boyle *et al.*, 1998). A molecular approach that would directly route an antigen into the MHC class I and II processing and presentation pathway might enhance its presentation to MHC class I-restricted $CD8^+$ T cells and class II-restricted $CD4^+$ T cells. Several studies have employed strategies targeting the MHC class I processing pathway to enhance the potency of DNA vaccines and generated enhanced $CD8^+$ T cell activities (Rodriguez *et al.*, 1997; Tobery and Siliciano, 1997; Wu and Kipps, 1997). Our study represents a successful approach that enhances the potency of DNA vaccines by targeting the MHC class II processing pathway. Other potential targeting strategies that may enhance MHC class II antigen presentation include using the intracellular sorting signals of the MHC class II-associated invariant chain (Ii) (Sanderson *et al.*, 1995) and using the intracellular sorting signals of the HLA-DM molecule (Lindstedt *et al.*, 1995). It will be interesting to generate chimeric molecules similar to the Sig/E7/LAMP-1 construct to see if such strategies will enhance the MHC class II presentation of cytoplasmic proteins such as HPV-16 E6 and E7.

The mechanisms by which DNA vaccines induce both immunologic and protective responses are under active investigation. Several studies have shown that bone marrow-derived antigen-presenting cells (APCs) are the most important cells for presenting the antigen to the immune system after both skin bombardment with a gene gun or intramuscular inoculation (Condon *et al.*, 1996; Casares *et al.*, 1997; Iwasaki *et al.*, 1997; Torres *et al.*, 1997). The effect of Sig/E7/LAMP-1 DNA vaccine most likely relies on the existence of the MHC class II pathway in the transfected professional antigen-presenting cells. Therefore, skin dendritic cells or hematopoietic professional APCs should play a direct role in the antigen processing in our system. The results of this study support our hypothesis that MHC class II^+ APCs are indeed the critical target for nucleic acid vaccines.

In the present study, we also observed enhanced E7-specific CTL activity and significantly increased E7-specific $CD8^+$ T cell precursors in mice vaccinated with Sig/E7/LAMP-1 DNA vaccine. There are several possible mechanisms that may account for the observed increase in the number of $CD8^+$ T cell precursors in mice vaccinated with Sig/E7/LAMP-1 DNA. First, direct priming by MHC class I presentation of E7 in antigen-presenting cells expressing Sig/E7/LAMP-1 is one plausible mechanism. We demonstrated that antigen-presenting cells expressing Sig/E7/LAMP-1 are capable of directly presenting E7 through the MHC class I pathway. Our data indicated that they may also be more efficient than wild-type E7 at presenting E7 through the MHC class I pathway. These data suggest that CTL epitopes from the chimeric Sig/E7/LAMP-1 product are presented on MHC class I molecules. Second, we speculate that enhanced CTL activity in C57BL/6 mice vaccinated with the chimeric Sig/E7/LAMP-1 DNA may also be due to an increase in $CD4^+$ T cell help as a result of improved presentation of MHC class II-restricted epitopes. In a previous study, we observed that CD4-knockout mice vaccinated with Sig/E7/LAMP-1 experienced a significant loss of CTL activity (Wu *et al.*, 1995). Finally, CTL activities generated by the Sig/E7/LAMP-1 DNA vaccine may have been contributed by the MHC class I presentation of exogenous proteins through a so-called "cross-priming effect" (Huang *et al.*, 1994). Although keratinocytes are unlikely to activate T cells directly, keratinocytes can take up and express DNA encoding foreign proteins such as E7. This subjects them to immunological attack, resulting in the release of the proteins. These proteins can in turn be taken up by professional APCs and presented through the MHC class I pathway. It is therefore possible that some of the CTL activities generated by Sig/E7/LAMP-1 DNA vaccination may be due to the cross-priming effect (Huang *et al.*, 1994).

In this study, we have also observed increased $CD4^+$ and $CD8^+$ T cell precursors in mice vaccinated with the Sig/E7 DNA vaccine. Although one may attribute the enhanced E7-specific cell-mediated immune responses in mice vaccinated with Sig/E7/LAMP-1 to the Sig/E7 portion, we believe that the mechanisms by which Sig/E7/LAMP-1 enhances E7-specific immune responses may be different from those of Sig/E7. First, in a previous study, we have observed that LB27 antigen-presenting cells expressing Sig/E7/LAMP-1 directly present E7 via the MHC class II pathway, while Sig/E7 is not so presented (Wu *et al.*, 1995). This observation indicates that Sig/E7/ LAMP-1 enhanced E7-specific $CD4^+$ T cell responses through a mechanism different from that of Sig/E7. Second, our more recent data indicate that dendritic cells transduced with Sig/E7 secrete E7 into the medium, while dendritic cells transduced with Sig/E7/LAMP-1 do not (T.-L. Wang, personal communication, 1999). Several studies have shown significant enhancement of humoral immune responses by adding signal peptide to a cytoplasmic protein (Haddad *et al.*, 1997; Inchauspe *et al.*, 1997). The earlier and greater E7-specific antibody responses that we observed in mice vaccinated with Sig/E7 compared with mice vaccinated with either wild-type E7 or Sig/E7/LAMP-1 (see Fig. 3) may be related to the secretion of E7. Since E7 was sent to different compartments in cells transduced with Sig/E7/LAMP-1 versus cells transduced with Sig/E7, the mechanisms by which these vaccines enhance E7-specific humoral and cell-mediated immune responses are most likely different.

Even though HPV-16 is associated with more that 50% of cervical cancers and their precursor lesions, there are many other types of HPV, such as HPV-18, -31, -33, and -45, that can be associated with cervical cancers and their precursor lesions. Thus, an ideal vaccine for HPV infections should cover most of these other types of HPV infections. Since many different kinds of DNA vaccines can be mixed and effectively administered together, DNA vaccines are clearly one of the best choices. Furthermore, highly pure, stable, DNA-based vaccines can be rapidly produced in large quantities.

While the Sig/E7/LAMP-1 DNA vaccine holds promise for mass immunization, two safety issues need to be resolved. First, the DNA may integrate into the host genome, resulting in the inactivation of tumor suppresser genes or the activation of oncogenes. This may lead to malignant transformation of the host cell. Fortunately, it is estimated that the frequency of integration is much lower than that of spontaneous mutation and integration should not pose any real risk (Nichols *et al.*, 1995). The second issue concerns potential risks associated with the presence of HPV-16 E7 protein in host cells. E7 is an oncoprotein that disrupts cell cycle regulation by binding to tumor suppressor pRB protein in nuclei (Lukas *et al.*, 1994). Thus, the presence of E7 in host cells may lead to accumulation of ge-

netic aberrations and eventual malignant transformation in the host cells. Sig/E7/LAMP-1 chimeric protein contains the sorting signal that delivers the protein into lysosomal and endosomal compartments, not the nucleus, and thus the likelihood of Sig/E7/LAMP-1 interacting with pRB in the nucleus is low. In addition, the oncogenicity of E7 can be further eliminated by introducing mutations into E7 DNA so that the resulting E7 protein cannot bind with pRB (Heck *et al.*, 1992) but still maintains most of its antigenicity.

In summary, our results indicate that linkage of the antigen gene to an endosomal/lysosomal targeting signal may greatly enhance the potency of nucleic acid vaccines. Specifically, we demonstrated that the Sig/E7/LAMP-1 DNA vaccine can generate strong antitumor immunity against HPV-16 E7-expressing tumors when administered intradermally. Therefore, Sig/E7/LAMP-1 DNA vaccines represent an exciting therapeutic approach for the control of HPV-associated malignancies and their precursors.

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